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Macrophilones from the Marine Hydroid *Macrorhynchia philippina* Can Inhibit ERK Cascade Signaling

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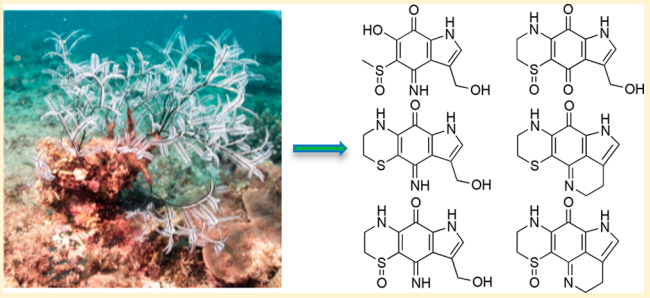
Supporting Information

ABSTRACT: Six new macrophilone-type pyrroloiminoquines were isolated and identified from an extract of the marine hydroid *Macrorhynchia philippina*. The proton-deficient and heteroatom-rich structures of macrophilones B−G (**2**−**7**) were elucidated by spectroscopic analysis and comparison of their data with those of the previously reported metabolite macrophilone A (**1**). Compounds **1**−**7** are the first pyrroloiminoquines to be reported from a hydroid. The macrophilones were shown to inhibit the enzymatic conjugation of SUMO to peptide substrates, and macrophilones A (**1**) and C (**3**) exhibit potent and selective cytotoxic properties in the NCI-60 anticancer screen. Bioinformatic analysis revealed a close association of the cytotoxicity profiles of **1** and **3** with two known B-Raf kinase inhibitory drugs. While compounds **1** and **3** showed no kinase inhibitory activity, they resulted in a dramatic decrease in cellular protein levels of selected components of the ERK signal cascade. As such, the chemical scaffold of the macrophilones could provide small-molecule therapeutic leads that target the ERK signal transduction pathway.



Hydroids are widely distributed in the world's oceans, but they have only sparsely been studied chemically compared to other colonial marine invertebrate organisms such as sponges, corals, and ascidians. However, previous chemical examinations of hydroids have afforded a diverse array of bioactive metabolites including anthracenes (anthracenones),[1−6] polyhalogenated monoterpenoids,[7] brominated *β*-carbolines,[8] piperidinols,[9] dithiocarbamates,[10,11] homoeicosanoids,[12] macrolactones,[13] a pentapeptide,[14] a 4-hydroxybenzoyl derivative,[15] and polyhydroxylated sterols.[16−18] Some of these metabolites exhibited significant cytotoxic activities;[1,13−15] thus hydroids represent an understudied resource of chemical diversity for potential anticancer discovery efforts. An extract from an Australian collection of the hydroid *Macrorhynchia philippina* showed significant activity in the NCI-60 cell line anticancer screen and, thus, was selected for detailed chemical investigation. According to the World Register of Marine Species the genus *Macrorhynchia* is synonymous with *Lytocarpus*,[19] and there is a report in the literature of 14-membered macrolides that were isolated from a hydroid described as *Lytocarpus philippinus*.[13] While there is some ambiguity in the literature concerning hydroid taxonomic descriptions and classification,[20,21] it is likely that the hydroid examined in the current study is the same as the hydroid that provided the macrolides. No macrolides were detected during fractionation of the *M. philippina* extract; rather, new pyrroloiminoquinone derivatives were isolated. We recently reported the structure elucidation and synthesis of macro-

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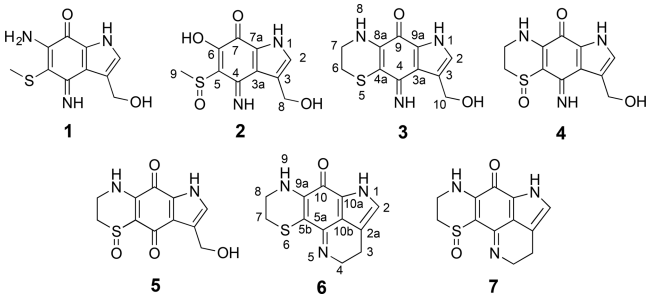
philone A (**1**), a potent cytotoxic metabolite that also arrests the small ubiquitin-like modifier (SUMO) conjugation cascade in vitro.[22] Post-translational attachment of the SUMO-protein to other protein substrates is an important regulatory component of critical cellular processes, and disruption of SUMOylation is often associated with cancer and other diseases.[23−25] Further investigation of the *M. philippina* extract provided six additional iminoquinone derivatives, macrophilones B−G (**2**−**7**), and their isolation, structural characterization, and biological evaluations are described below.



RESULTS AND DISCUSSION

Sequential chromatography of the *M. philippina* extract on DIOL and Sephadex LH-20 supports, followed by repeated C_{18} reversed-phase HPLC, provided macrophilone A (**1**)[22] along with six additional structural analogues (**2**−**7**). These compounds all have a high heteroatom content and relatively few protons observable by NMR, so the total synthesis of **1** was undertaken to confirm its structure.[22] The molecular formula of macrophilone B (**2**) was established as $C_{10}H_{10}N_2O_4S$ based on HRESIMS data, and this differed from the molecular formula of **1** by the addition of two oxygens and the loss of NH. The ^{1}H (Table 1) and ^{13}C NMR data (Table 2) of **2** corresponded closely with those of **1**, showing all the characteristic signals for a hydroxymethyl-substituted pyrroloiminoquinone moiety. A full suite of HMBC correlations, including correlations from H_2-8 (δ_H 4.69 s) to the C-4 imino carbon (δ_C 160.3) and H-2 (δ_H 7.22 s) to the C-7 oxo carbon (δ_C 170.2), supported this assignment (Figure 1). However, the UV spectrum of **2**, with absorbance maxima at 212, 233, 257, 310, and 359 nm, differed from the spectrum recorded for **1**, which indicated a modified chromophore. A notable difference in the ^{13}C NMR spectrum of **2** was the loss of a signal for an amino-bearing carbon (δ_C 156.4 in **1**) and the appearance of a signal for an oxygenated sp^2 carbon (δ_C 173.1, C-6), which suggested that the amino group in **1** was replaced by a hydroxy group in **2**. In addition, significant deshielding was observed for both the sulfur-bearing methyl (δ_C 37.9 in **2**; 17.1 in **1**) and C-5 sp^2 (δ_C 101.2 in **2**; 95.7 in **1**) carbons. These data and molecular formula considerations indicated the thioether in **1** was oxidized to a sulfoxide in **2**. An HMBC correlation from H_3-9 (δ_H 2.97 s) to C-5 showed the position of the sulfoxide was consistent with that of the thiomethyl group in **1**, and this was supported by an additional four-bond correlation to C-6 when the HMBC experiment was optimized for 2 Hz couplings (Figure 1). Thus, macrophilone B (**2**) was elucidated as 6-hydroxy-3-(hydroxymethyl)-4-imino-5-(methylsulfinyl)-1,4-dihydro-7*H*-indol-7-one.

Macrophilone C (**3**) had a molecular formula of $C_{11}H_{11}N_3O_2S$ as determined by HRESIMS data, requiring seven degrees of unsaturation. The UV spectrum of **3** with absorptions at 213, 259, 325, and 390 nm was similar to the UV spectrum of **1**, but its molecular formula revealed one additional carbon and an additional unsaturation equivalent. The ^{1}H NMR spectrum of **3** in CD_3OD showed signals for an olefinic proton (δ_H 7.20 s, H-2), an oxymethylene (δ_H 4.70 s, H_2-10), and two mutually coupled methylenes (δ_H 3.86 t, J = 4.9 Hz, H_2-7; 3.04 t, J = 4.9 Hz, H_2-6). Numerous efforts to observe exchangeable protons in **3** by acquiring spectra in dry DMSO-d_6 were unsuccessful. The ^{13}C NMR spectrum in CD_3OD displayed seven nonprotonated sp^2 carbons [δ_C 167.2 (C-9), 159.6 (C-4), 144.6 (C-8a), 128.1 (C-9a), 125.5 (C-3), 121.6 (C-3a), 91.6 (C-4a)], a protonated sp^2 (δ_C 126.1, C-2), and a hydroxymethylene carbon (δ_C 55.7, C-10), which corresponded closely with the pyrroloiminoquinone skeleton of **1**.[22] Two additional methylene carbons (δ_C 43.4, C-7; 22.3, C-6) and the requirement of one more unsaturation equivalent indicated they bridged the amino and thio groups to form a 3,4-dihydro-2*H*-1,4-thiazine ring. The deshielded chemical shifts of H_2-7 (δ_H 3.86) and C-7 (δ_C 43.4) in comparison with those of H_2-6 (δ_H 3.04) and C-6 (δ_C 22.3) suggested that C-7 was N-substituted and C-6 was S-substituted. HMBC

Table 1. ^{1}H NMR Data, δ_H (J in Hz), for Compounds 2−7[a]

pos.	2	3	3[b]	4	5	6	7
2	7.22, s	7.20, s	7.09, s	7.38, s	7.26, s	7.07, s	7.25, s
3						2.92, t (7.5)	2.98, m
4						3.89, t (7.5)	4.00, m
6		3.04, t (4.9)	2.87, t (4.7)	3.42, br d (14.0) 2.76, td (14.0, 4.1)	3.21, dt (14.1, 2.7) 2.54, td (14.1, 3.9)		
7		3.86, t (4.9)	3.56, t (4.7)	4.07, br d (15.6) 3.96, br t (14.0)	3.95, ddd (14.9, 3.9, 2.7) 3.74, td (14.6, 2.7)	3.01, t (4.9)	3.33, m 2.69, td (14.0, 3.7)
8	4.69, s					3.86, t (4.9)	4.07, m 3.95, m
9	2.97, s						
10		4.70, s	4.48, s	4.76, s	4.77 s		

[a]600 MHz, CD_3OD. [b]Data recorded in DMSO-d_6.

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Table 2. ^{13}C NMR Data, δ_C, Type, for Compounds 2−7[a]

pos.	2	3	3[b]	4	5	6	7
2	126.3, CH	126.1, CH	124.9, CH	127.6, CH	126.8, CH	125.0, CH	126.4, CH
2a						118.8, C	119.6, C
3	124.4, C	125.5, C	125.6, C	126.2, C	127.6, C	18.1, CH_2	17.9, CH_2
3a	122.7, C	121.6, C	125.5, C	122.1, C	124.2, C		
4	160.3, C	159.6, C	161.0, C	160.2, C	179.5, C	44.2, CH_2	45.1, CH_2
4a		91.6, C	102.1, C	100.1, C	106.7, C		
5	101.2, C						
5a						155.3, C	155.2, C
6	173.1, C	22.3, CH_2	23.7, CH_2	39.6, CH_2	39.8, CH_2		
6a						90.6, C	98.7, C
7	170.2, C	43.4, CH_2	42.3, CH_2	30.3, CH_2	30.3, CH_2	22.1, CH_2	39.5, CH_2
7a	130.2, C						
8	55.7, CH_2					43.6, CH_2	30.8, CH_2
8a		144.6, C	137.8, C	147.0, C	147.0, C		
9	37.9, CH_3	167.2, C	168.6, C	166.6, C	168.2, C		
9a		128.1, C	127.6, C	129.0, C	128.7, C	146.0, C	148.7, C
10		55.7, CH_2	56.4, CH_2	55.7, CH_2	56.2, CH_2	166.1, C	165.2, C
10a						123.4, C	124.3, C
10b						122.4, C	121.7, C

[a]150 MHz, CD_3OD. [b]Data recorded in DMSO-d_6.

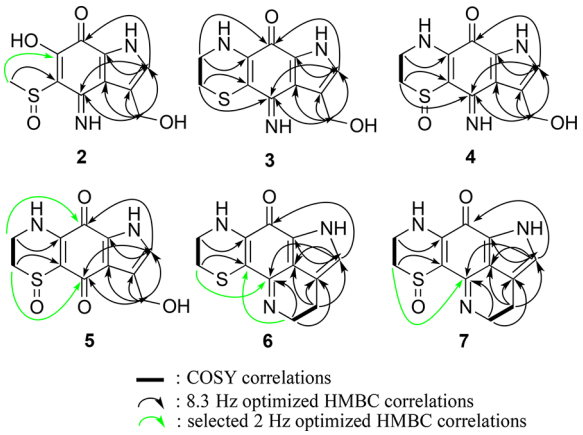

Figure 1. Key COSY and HMBC correlations for compounds **2**−**7**.

correlations (Figure 1), including those from H_2-10 to C-2/C-3/C-3a/C-4 and H-2 to C-9a/C-9, established the relative orientation of the pyrroloiminoquinone rings in **3**. HMBC correlations from H_2-6 to C-4/C-4a and from H_2-7 to C-8a/C-9 confirmed the presence and regiochemistry of the fused thiazine ring. Thus, the structure of macrophilone C (**3**) was established as the thiazine ring analogue of **1**.

Macrophilone D (**4**) was assigned a molecular formula of $C_{11}H_{11}N_3O_3S$ from its HRESIMS data, revealing one additional O atom in the molecule compared with **3**. The highly analogous nature of the NMR data, including the multiplicity of all 11 carbons, suggested that **4** was a heteroatom-oxide derivative of **3**. ^{13}C NMR data of **4** had signals attributed to the hydroxymethyl pyrrole moiety, carbonyl, and imino carbons as in **3**, indicating that **4** had the same pyrroloiminoquinone substructure. This was supported by HMBC correlations as depicted in Figure 1 from H_2-10 (δ_H 4.76 s) to C-2 (δ_C 127.6, CH), C-3 (δ_C 126.2), C-3a (δ_C 122.1), and imino carbon C-4 (δ_C 160.2) and from the olefinic proton H-2 (δ_H 7.38 s) to C-9a (δ_C 129.0), C-4, and carbonyl carbon C-9 (δ_C 166.6), which were very similar to those observed with **3**. Thus, the remaining oxygen atom in the molecule should be assigned to a heteroatom of the 1,4-thiazine ring. Significant deshielding (Δ +8.5 ppm) was observed for the ^{13}C NMR signal of the sulfur-bearing sp^2 carbon in **4** (δ_C 100.1 in **4**) compared with that of **3** (δ_C 91.6) indicated the remaining oxygen was bound to sulfur to form a sulfoxide. COSY correlations between H_2-6 (δ_H 3.42 br d, J = 14.0 Hz; 2.76 td, J = 14.0, 4.1 Hz) and H_2-7 (δ_H 4.07 br d, J = 15.6 Hz; 3.96 br t, J = 14.0 Hz), in combination with HMBC correlations from H_2-6 and H_2-7 to the two nonprotonated carbons C-4a (δ_C 100.1) and C-8a (δ_C 147.0), confirmed the presence of a 1-oxo-3,4-dihydro-2*H*-1,4-thiazine moiety. A four-bond correlation from H_2-6 to the imino carbon C-4 provided further support that the regiochemistry of the 1,4-thiazine moiety in **4** was identical to that in **3**. This established macrophilone D (**4**) as the sulfoxide derivative of **3**.

Macrophilone E (**5**) had a molecular formula of $C_{11}H_{10}N_2O_4S$ based on HRESIMS data, which required eight degrees of unsaturation. The ^1H and ^{13}C NMR spectra were very similar to those of **4**. The only notable difference was the replacement of the imino carbon signal (δ_C 160.2 in **4**) by a carbonyl carbon signal at δ_C 179.5 and the deshielded chemical shift of the sulfur-bearing sp^2 carbon signal (δ_C 100.1 in **4**; 106.7 in **5**), indicating that **5** was the quinone equivalent of **4**. This assignment was supported by COSY and HMBC correlations that were very similar to those observed with **4**, which included four-bond correlations from H_2-6 (δ_H 3.21 dt, J = 14.1, 2.7 Hz; δ_H 2.54 td, J = 14.1, 3.9 Hz) and H_2-7 (δ_H 3.95 ddd, J = 14.9, 3.9, 2.7 Hz; δ_H 3.74 td, J = 14.6, 2.7 Hz) to the carbonyl carbons C-4 (δ_C 179.5) and C-9 (δ_C 168.2, C-9), respectively, and from H_2-10 (δ_H 4.77 s) to C-4 (Figure 1). In addition, the regiochemistry of the 1,4-thiazine and pyrrole rings was indicated by the diagnostic deshielded (C-4) and shielded (C-9) chemical shifts of the quinone carbonyl carbons.[26,27] Macrophilone E (**5**) was thus established as the quinone analogue of **4**.

Macrophilone F (**6**) had a molecular formula of $C_{12}H_{11}N_3OS$ as determined by HRESIMS data, implying nine degrees of unsaturation. The ^{13}C NMR spectrum displayed 12 carbon signals including eight characteristic sp^2

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carbons for the pyrroloiminoquinone core as described in **3** and four methylene carbons (Table 2). The ^{1}H NMR spectrum showed signals for a pyrrolo proton (δ_H 7.07 s, H-2) and four aliphatic methylenes (δ_H 3.89 t, J = 7.5 Hz, H_2-4; 2.92 t, J = 7.5 Hz, H_2-3; 3.86 t, J = 4.9 Hz, H_2-8; 3.01 t, J = 4.9 Hz, H_2-7), which constituted two separate proton−proton spin systems by J-coupling and COSY analysis. HMBC correlations (Figure 1) from H_2-4 to the imino carbon C-5a (δ_C 155.3) and from H_2-3 to the three olefinic carbons C-2 (δ_C 125.0, CH), C-2a (δ_C 118.8, C), and C-10b (δ_C 122.4, C) revealed that **6** had a pyrrolo[4,3,2-*de*]quinoline skeleton found in makaluvamines A−F, which were isolated from sponges of the genus *Zyzzya*.[28] Comparison of the NMR data of **6** with those of **3**, in combination with HMBC correlations observed from the mutually coupled methylenes H_2-7 and H_2-8 to the olefinic carbons C-6a (δ_C 90.6, C) and C-9a (δ_C 146.0, C), respectively, suggested the presence of the same 1,4-thiazine moiety in **6**. Furthermore, four-bond HMBC correlations from H_2-4 to C-6a and from H_2-7 to C-5a confirmed that the regiochemistry of the 1,4-thiazine was the same as that in **3**. The structure of macrophilone E was assigned as the tetracyclic pyrroloiminoquinone **6**.

The molecular formula of macrophilone G (**7**) was determined to be $C_{12}H_{11}N_3O_2S$ on the basis of HRESIMS data, consistent with an extra O atom in comparison with that of **6**. This suggested that **7** was likely an oxidized derivative of **6**. The ^{13}C NMR data for **7** were very similar to those recorded for **6**, except for significant deviation of the signals for carbons in the 1,4-thiazine moiety. Close correspondence between the ^{13}C NMR data for the 1,4-thiazine moiety in **7** with those in **2** suggested that **7** was the sulfoxide derivative of **6**. All of the observed COSY and HMBC correlations were consistent with the oxothiazine structure assigned for macrophilone G (**7**).

Compounds **2**, **4**, **5**, and **7** contain an unsymmetrical sulfoxide group; however they were isolated as optically inactive compounds, and no Cotton effect was observed in their electronic circular dichroism (ECD) spectra, which indicated that the natural sulfoxides were racemic. While pyrroloiminoquinones have been isolated from numerous marine sponges and ascidians, macrophilones A−G (**1**−**7**) are the first of this metabolite class to be described from a hydroid. No other naturally occurring iminoquinones fused with the 3-hydroxymethyl pyrrole ring in **1**−**4** and/or the 1,4-thiazine moiety in **3**, **4**, **6**, and **7** have been reported. The broad taxonomic distribution of pyrroloiminoquinones, from the most primitive multicellular organisms (sponges) to some of the most advanced invertebrates (ascidians), is suggestive that this class of metabolite has a microbial biosynthetic origin. Macrophilone A (**1**) was previously shown to inhibit SUMO conjugation via an oxidative mechanism that results in thiol cross-linking of proteins in the SUMOylation cascade.[22] Treatment with **1** leads to an increase in reactive oxygen species (ROS) in cells, but it also demonstrated potent cytotoxic activity that was not related to ROS or general oxidative stress. Compounds **2**−**7** were also evaluated for their ability to block SUMOylation of a substrate peptide in an in vitro electrophoretic mobility shift assay.[29,30] They showed relatively modest inhibitory activity in the SUMO assay with IC_{50} values that ranged from 11.9 μM for **2** to >100 μM for **6** (Supporting Information). To further explore their cytotoxic properties, macrophilones A (**1**), C (**3**), and D (**4**) were tested in the NCI-60 cell line screening assay. A pattern of selective cytotoxicity at the GI_{50} level (concentration for 50% growth inhibition) was evident for these compounds (Supporting Information). Bioinformatic analysis using the CellMiner Web-based platform (https://discover.nci.nih.gov/cellminer/)[31] showed that compounds **1** and **3** highly correlated with dabrafenib[32] and vemurafenib,[33] two FDA-approved B-Raf inhibitors, in addition to other B-Raf targeting agents that are still undergoing clinical trials (Supporting Information). Dabrafenib and vemurafenib were approved as kinase inhibitors for the treatment of metastatic melanoma patients with the V600E mutation in their *b-raf* gene. Macrophilones A (**1**) and C (**3**) were most potent in suppressing growth of cancer cell lines that harbor the B-Raf^{V600E} mutation, particularly the Colo205 colon line and the MALM-3M, SK-MEL-5, and MDA-MB-435 melanoma cell lines. The B-Raf^{V600E} mutation results in a dramatic increase in B-Raf kinase activity that is independent of upstream stimuli, and this leads to constitutive activation of the downstream MEK-ERK signaling pathway and uncontrolled cell growth and survival.[34,35] While dabrafenib and vemurafenib have demonstrated clinical efficacy in treating melanoma and other carcinomas that arise from aberrant B-Raf signaling, disease relapse is a major problem, and alternative inhibitors of the Ras-Raf-MEK-ERK signal transduction pathway are needed for therapeutic development. Compounds **1**, **3**, and **4** were evaluated for their ability to inhibit the kinase catalytic activity of B-Raf, C-Raf, MEK, and ERK, but no reduction in kinase activity was observed (data not shown). However, after an 18 h treatment of Colo205 cells with these macrophilones, Western blot analysis revealed a dramatic and selective reduction in the cellular levels of key proteins in the pathway. Treatment with 10 μM **1**, **3**, or **4** resulted in striking reductions of the total protein levels of B-Raf, C-Raf, and MEK, but there was no corresponding loss of ERK protein levels (Figure 2). Macrophilone A (**1**) was also effective at reducing these proteins at 1 μM, while **3** and **4** were not active at this lower concentration. In fact, treatment with 1 μM of compounds **3** and **4** resulted in increased phosphorylation (activation) of both MEK and ERK. While the exact cause of this biphasic response is unclear, the slight gel-shift observed for C-Raf is due to ERK-mediated feedback phosphorylation of multiple serine and threonine residues in C-Raf.[36] In contrast, the positive control SB-590885, which is a Raf kinase inhibitor, blocked phosphorylation of MEK and subsequent activation of ERK, but it had no significant impact on any of the total protein concentrations in the pathway. Thus, the macrophilones apparently abolish signaling in the Ras-Raf-MEK-ERK cascade in a concentration- and time-dependent manner (Supporting Information) by significantly lowering the cellular levels of specific kinases that are required to activate key downstream effectors in the pathway.

In summary, we report the isolation and structural assignment of macrophilones B−G (**2**−**7**). They were obtained from a marine hydroid, which now expands the known phylogenetic distribution of marine pyrroloiminoquinones to include the class hydrozoa. Similar to prior findings with macrophilone A (**1**), compounds **2**−**7** showed general inhibitory activity in an in vitro SUMOylation assay. In addition, significant cytotoxicity to cancer cell lines was also observed for a number of these metabolites. The selective cytotoxicity profiles of macrophilones A (**1**) and C (**3**) in the NCI-60 screen showed increased potency toward cell lines harboring the V600E mutation in B-Raf kinase. Bioinformatic analysis revealed a close correlation between the cytotoxicity

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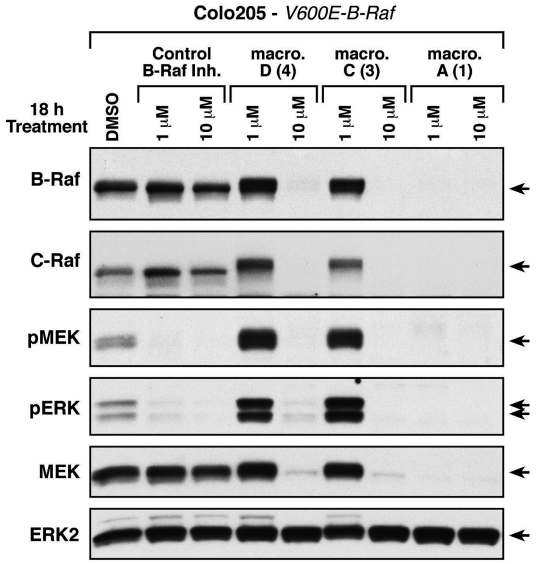

Figure 2. Western blot analysis of the effect of macrophilones A (**1**), C (**3**), and D (**4**) on the ERK signaling cascade. Antibodies to B-Raf, C-Raf, MEK, and ERK2 total protein, as well as phosphorylated (activated) pMEK and pERK, were used in the analysis. Treatment of Colo205 cells that harbor the V600E B-Raf mutation with 10 μM of compounds **1**, **3**, or **4** for 18 h dramatically reduced cellular levels of B-Raf, C-Raf, and MEK but had no impact on ERK levels. The same effect was seen with macrophilone A (**1**) at 1 μM, but not with macrophilones C (**3**) or D (**4**) at 1 μM. The control compound SB-590885 is a Raf kinase inhibitor that disrupts signaling by reducing MEK and subsequent ERK phosphorylation, but it had no effect on the nonphosphorylated protein levels.

profiles of **1** and **3** with two kinase inhibitors approved by the FDA for treatment of B-Raf^{V600E}-associated cancers. Although **1** and **3** had no direct kinase inhibitory activity, they resulted in striking reductions in cellular Raf and MEK protein levels. Loss of these components of the ERK cascade represents an alternative approach to abrogating signal transduction via this pathway. Compounds that reduce key protein levels, rather than act as competitive kinase inhibitors, may provide a complementary means to target this pathway for possible therapeutic applications.

EXPERIMENTAL SECTION

General Experimental Procedures. UV and IR spectra were measured with a Varian Cary 50 UV/vis spectrophotometer and a PerkinElmer Spectrum 2000 FT-IR spectrometer, respectively. NMR spectra were obtained with a Bruker Avance III NMR spectrometer equipped with a 3 mm cryogenic probe and operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C. Spectra were calibrated to residual solvent signals at δ_H 3.31 and δ_C 49.0 (CD_3OD) and δ_H 2.50 and δ_C 39.5 (DMSO-d_6). Preparative reversed-phase HPLC was run on a Varian PrepStar preparative HPLC system using a Phenomenex Jupiter C_{18} (5 μm, 300 Å, 250 × 10 mm) column run with the indicated gradient. (+)HRESIMS data were acquired on an Agilent Technology 6530 Accurate-mass Q-TOF LC/MS.

Animal Material. Specimens of the hydroid *Macrorhynchia philippina* were collected in Northwestern Australia in August 1988, under contract through the Coral Reef Research Foundation for the Natural Products Branch, National Center Institute. Taxonomic identification of the hydroid was done by Jeanette E. Watson, Museum of Victoria, Melbourne, Australia, and a voucher specimen (voucher ID Q66C1539; NSC C004385) was deposited at the Smithsonian Institute, Washington, DC.

Extraction and Isolation. The hydroid specimen (165 g, dry weight) was extracted according to the procedures detailed by McCloud to give 3.75 g of organic solvent (CH_2Cl_2–MeOH, 1:1, and 100% MeOH) extract.[37] A portion of the organic extract (2.01 g) was fractionated on Diol SPE cartridges (2 g) eluting with 9:1 hexane–CH_2Cl_2 (fraction A, 680.2 mg), 5:1 CH_2Cl_2–EtOAc (fraction B, 304.4 mg), 100% EtOAc (fraction C, 52.7 mg), 5:1 EtOAc–MeOH (fraction D, 62.0 mg), and 100% MeOH (fraction E, 521.9 mg) in a stepwise manner. Fractions D and E showed significant cytotoxicity against two colon cancer cell lines and thus were combined and dissolved in 3 mL of 1:1 CH_2Cl_2–MeOH. The soluble portion of the mixture (429.0 mg) was chromatographed on a Sephadex LH-20 column (25 × 800 mm), using 1:1 CH_2Cl_2–MeOH as eluent, to obtain 14 fractions. The active fractions (G–K) were purified individually by reversed-phase C_{18} HPLC using a linear gradient elution of MeCN–H_2O (10:90–50:50, containing 0.2% formic acid) over 30 min to afford **1** (7.2 mg), **3** (21.0 mg), **6** (1.3 mg), and two impure fractions containing **2** and **5**, respectively, as well as a mixture of **4** and **7**. Further purification was effected by reversed-phase C_{18} HPLC using a linear gradient elution of MeOH–H_2O (5:95–40:60, containing 0.2% formic acid) over 30 min to yield **2** (0.5 mg), **4** (5.3 mg), **5** (0.4 mg), and **7** (1.4 mg).

*Macrophilone B (**2**):* optically inactive orange solid; UV (MeOH) λ_{max} (log ε) 212 (3.94), 233 (4.00), 257 (4.00), 310 (3.76), 359 (3.80); IR (KBr) ν_{max} 3272 (br), 2924, 2853, 1667, 1585, 1504, 1455, 1414, 1362 cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; HRESIMS m/z 255.0428 [M + H]$^+$ (calcd for $C_{10}H_{11}N_2O_4S$, 255.0440).

*Macrophilone C (**3**):* green solid; UV (MeOH) λ_{max} (log ε) 213 (3.84), 259 (3.94), 325 (3.76), 390 (sh); IR (KBr) ν_{max} 3127 (br), 2956, 1672, 1616, 1578, 1508, 1330 cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; HRESIMS m/z 250.0646 [M + H]$^+$ (calcd for $C_{11}H_{12}N_3O_2S$, 250.0650).

*Macrophilone D (**4**):* optically inactive orange solid; UV (MeOH) λ_{max} (log ε) 218 (4.12), 255 (sh), 337 (3.97), 446 (2.95); IR (KBr) ν_{max} 3313 (br), 2920, 1646, 1574, 1551, 1499, 1341, 1181 cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; HRESIMS m/z 266.0593 [M + H]$^+$ (calcd for $C_{11}H_{12}N_3O_3S$, 266.0599).

*Macrophilone E (**5**):* optically inactive yellow solid; UV (MeOH) λ_{max} (log ε) 228 (4.05), 248 (sh), 313 (3.71), 357 (3.72); IR (KBr) ν_{max} 3276 (br), 2924, 1668, 1622, 1582, 1362 cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; HRESIMS m/z 267.0436 [M + H]$^+$ (calcd for $C_{11}H_{11}N_2O_4S$, 267.0440).

*Macrophilone F (**6**):* green solid; UV (MeOH) λ_{max} (log ε) 212 (3.69), 263 (3.82), 337 (3.61), 405 (sh); IR (KBr) ν_{max} 3230 (br), 2930, 1664, 1616, 1594, 1539, 1353 cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; HRESIMS m/z 246.0696 [M + H]$^+$ (calcd for $C_{12}H_{12}N_3OS$, 246.0701).

*Macrophilone G (**7**):* optically inactive orange solid; UV (MeOH) λ_{max} (log ε) 218 (4.01), 341 (3.88), 447 (3.13); IR (KBr) ν_{max} 3240 (br), 2924, 2853, 1665, 1613, 1569, 1352 cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 1 and 2; HRESIMS m/z 262.0643 [M + H]$^+$ (calcd for $C_{12}H_{12}N_3O_2S$, 262.0650).

Biological Characterization. Inhibition of the SUMO conjugation enzymatic cascade was assessed using a fluorescently tagged model substrate peptide following the procedures previously reported.[29,30] DMSO solutions of macrophilones A (**1**), C (**3**), and D (**4**) were tested for cytotoxicity against 60 human tumor cell lines in the NCI-60 cell screening assay, and the results were analyzed using bioinformatics tools on the publicly accessible CellMiner Web site (https://discover.nci.nih.gov/cellminer/).[31] Western blot analysis used antibodies to B-Raf, C-Raf, and ERK2 from Santa Cruz Biotechnology, antibodies to pS217/221-MEK and pT202/Y204-ERK from Cell Signaling Technologies, antibodies to pERK from Sigma, and antibodies to MEK1 from BD Biosciences. Colo205 cells were cultured at 37 °C under 5% CO_2 in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Compounds **1**, **3**, and **4** and Raf inhibitor SB-590885 were added to culture medium dissolved in DMSO (final concentration in the assays was 0.1% v/v); controls received vehicle only. Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed

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under stringent conditions using radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 10% glycerol, 1% NP-40 alternative, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.15 U/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium vanadate, 20 μM leupeptin). Lysates were clarified by centrifugation and equalized for protein content, prior to analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

To monitor the effect of the compounds **1**, **3**, and **4** on Raf kinase activity, purified kinase-active Raf proteins were added to 10 μL of 30 mM Tris [pH 7.4] containing 10 μM of the indicated compound/drug and incubated at room temperature for 20 min, prior to the addition of 40 μL of kinase buffer (30 mM Tris [pH 7.4], 1 mM dithiothreitol [DTT], 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM ATP) containing 20 μCi of [γ^{32}P]ATP and 0.1 μg of kinase-inactive MEK. To evaluate the effect on MEK1 kinase activity, purified WT MEK1 proteins were incubated with the compounds/drugs as indicated above, prior to the addition of 40 μL of kinase buffer containing 20 μCi of [γ^{32}P]ATP and 0.1 μg of kinase-inactive ERK2. All kinase reactions were incubated at 30 °C for 30 min, following which the assays were terminated by the addition of gel sample buffer (250 mM Tris [pH 6.8], 50 mM DTT, 10% SDS, 30% glycerol). The samples were then analyzed by SDS-PAGE and autoradiography.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00343.

Experimental procedures, additional figures, and full spectroscopic data for all new compounds (PDF)

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